SPECIES DIFFERENCES IN RATES OF OSMOTIC HEMOLYSIS WITHIN THE GENUS PEROMYSCUS *

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Introduction

That definite species differences exist in the properties of the red cell membrane has been recognized at least since the studies of Rywosch in 1907. The possible significance of such specific differences in regard to zoological classification and animal identification has been pointed out by Jacobs (1931). Investigation of the rates of osmotic hemolysis in approximately 50 species of vertebrates led to the conclusion that "not only may individual species be identified but frequently unmistakable evidences of zoological relationship may be traced throughout a group of similar forms." In 1938 Jacobs and collaborators demonstrated striking differences in the permeability properties of the erythrocytes of the rat and mouse representing closely related genera. The purpose of the present investigation was to demonstrate measurable and consistent differences in the rates of hemolysis among a number of species within the genus Peromyscus.

Materials and Methods

The mice used in this investigation consisted of four species representing different degrees of taxonomic relationship (Miller, 1923) from widely separated geographical regions as follows:

Subgenus Haplomylomys Osgood
P. eremicus fraterculus—La Jolla, California
Subgenus Peromyscus Gloger
Species group—leucopus
P. leucopus noveboracensis—Vermont; Moville, Iowa
P. gossypinus palmarius—Sebring, Florida
Species group—truei
P. truei truei—Deadman Flat, Arizona

In addition, the guinea pig (*Cavia cobaya*) representing a distantly related rodent species was used for purposes of contrast.

Blood was obtained from each mouse under light ether anesthesia by cardiac puncture after the method of Hicks and Little (1931). About 0.5 cc. could be removed from a mouse without fatality. The blood was immediately expressed into a small beaker containing about 10 cc. of 0.9 per cent saline and defibrinated by stirring. The suspension was then washed down by centrifuge and the cells restored to the original blood volume with saline.

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The substances employed in the hemolysis studies were 0.3 molar solutions in distilled water of non-electrolytes including ethylene glycol, glycerol and erythritol representing progressively larger polyhydric alcohol molecules, and thiourea.

The method of determining rates of hemolysis was essentially that described by Jacobs (1930). To 5 cc. of one of the above solutions in a test tube in a water bath maintained at 20° C. was quickly added one drop of blood on a specially prepared plunger which simultaneously stirred the cells, producing an even suspension. With the aid of a stop-watch the time for 75 per cent hemolysis of the cells was determined by comparison with a standard suspension (one drop of the same blood in 20 cc. of saline) in a test tube adjacent to that containing the hemolysing suspension. This comparison was effected by means of a thin band of light viewed through the test tubes. Approximately 75 per cent hemolysis was attained when the band of light was visible in the hemolysing suspension to the same degree as in the standard. In practice the blood to be tested was so

TABLE I

Species differences in rates of osmotic hemolysis

		Time in seconds for 75 per cent hemolysis at 20° C. in 0.3M											
		Ethylene glycol			Glycerol			Erythritol			Thiourea		
Species	No.	Low	High	Ave.	Low	High	Ave.	Low	High	Ave.	Low	High	Ave.
P. leucopus	17	4.7	6.4	5.6	7.0	12.3	9.5	20.6	49.0	31.8	10.7	15.3	13.3
P. gossypinus	14	5.6	7.8	7.1	15.0	28.5	22.1	47.0	195.0	110.0	13.3	21.7	19.4
P. truei	6	6.8	8.0	7.3	33.6	44.3	39.2	150.0	250.0	193.0	28.7	36.0	32.1
P. eremicus	15	5.6	6.7	6.0	31.0	58.5	44.3	150.0	465.0	259.0	16.8	28.5	23.3
Cavia cobaya	3	10.6	15.4	13.6	130.0	223.0	178.0	>30 hrs. <42 hrs.			116.0	143.0	126.0

adjusted with saline that the band of light was just barely visible through the standard suspension since this offered the most easily recognized end point.

In performing the experiments test tubes were carefully chosen for uniformity, standard suspensions were prepared as soon as the blood samples were obtained, and hemolysis rates were determined immediately. All tests were performed in duplicate whenever possible. Remaining portions of blood samples were kept in refrigeration storage at approximately 4° C. Except for certain storage experiments where pooled blood was used, hemolysis rates were obtained with erythrocytes from individual animals.

EXPERIMENTAL RESULTS

The method of determining rates of hemolysis as described above was very simple and apparently crude, but with proper care the results of tests performed in duplicate proved to be markedly consistent. Variation in duplicate measurements of the time for 75 per cent hemolysis of the red cells in any one of the solutions rarely exceeded 10 per cent and most often was less than 5 per cent. With practice, especially in preparing suitable standard suspensions, duplication

was brought to within 2 per cent. It was reasonable to assume, therefore, that the differences in hemolysis times obtained here between one species and another represented true specific differences.

The times for 75 per cent hemolysis of the erythrocytes of the species investigated are summarized in Table I. Evidence of zoological relationship is readily apparent. When compared with the rate of hemolysis of guinea pig (Cavia) erythrocytes, the hemolysis rates of all the Peromyscus erythrocytes appear to be of the same order of magnitude. With erythritol, for example, the difference

TABLE II

Comparison of glycerol and thiourea times and G/T ratios of four species in the genus Peromycus (temperature 20° C.)

$$G/T\ ratio = \frac{hemolysis\ time\ in\ glycerol}{hemolysis\ time\ in\ thiourea}$$

	Time in seconds for 75 per cent hemolysis		
	0.3M Glycerol	0.3M Thiourea	G/T Ratio
P. leucopus	12.2	13.8	0.88
	8.1	13.7	0.59
	12.3	15.3	0.80
	7.0	10.7	0.65
	Ave. 9.5	13.3	0.71
P. gossypinus	28.5	21.4	1.33
	22.5	20.8	1.08
	24.4	21.7	1.12
	15.0	13.3	1.13
	Ave. 22.1	19.4	1.14
P. truei	44.3	33.2	1.33
	33.6	29.8	1.13
	43.4	36.0	1.21
	35.6	28.7	1.24
	Ave. 39.2	32.1	1.22
P. eremicus	58.5	27.3	2.11
	40.2	22.9	1.76
	54.6	27.5	1.98
	31.0	16.8	1.85
	Ave. 44.4	23.3	1.90

in hemolysis time between leucopus cells and eremicus cells (of the order 1 : 8) is small when compared with the difference between eremicus cells and guinea pig cells (1 : 540). On the other hand, consistent differences in hemolysis rates among the species within the genus are demonstrable. Leucopus cells are most readily hemolysed by each of the permeating substances; gossypinus cells are hemolysed at a somewhat slower rate. Generally truei and eremicus cells are hemolysed less rapidly than either leucopus or gossypinus cells. It is interesting to note in this regard that leucopus and gossypinus are placed taxonomically within the same species group.

The rates of osmotic hemolysis in glycerol especially often reveal striking specific differences and sometimes offer evidence of relationship (Jacobs, 1931; 1938). From Table I it can be seen that all the Peromyscus red cells attain the condition of 75 per cent hemolysis in less than one minute. Yet the hemolysis times for the red cells of each species are apparently confined to definite limits within this time.

According to Jacobs and associates (1938), comparison of the rates of osmotic hemolysis in glycerol and thiourea within a species may provide an index for species identification. Table II records in the first two columns the hemolysis times in glycerol and in thiourea respectively for each species of mouse investigated. The figures in the third column (G/T ratio) are obtained by dividing the glycerol hemolysis time by the thiourea hemolysis time. The data have been selected to show the extent of variation found in each species. The average figure for each species is the arithmetic mean of the results for all individuals

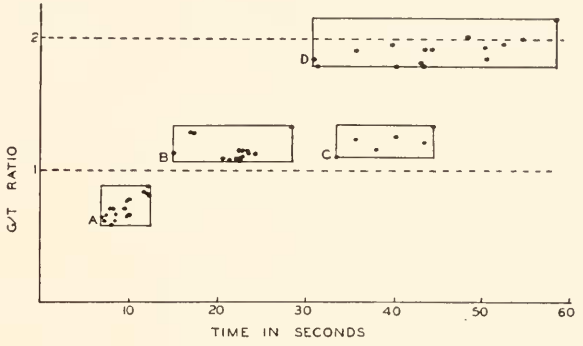

FIGURE 1. Species differentiation by osmotic hemolysis. Each dot represents an individual plotted along the abscissa in terms of the time for 75 per cent hemolysis in 0.3 molar glycerol at 20° C. and along the ordinate in terms of the G/T ratio. The hollow rectangles represent different species:

A = P. leucopus B = P. gossypinus C = P. truei
D = P. eremicus

studied within each species. It is evident from the table that the glycerol/thiourea ratio is constant for each species within fairly narrow limits. Leucopus which has the lowest ratio and eremicus which has the highest ratio are readily separated from gossypinus and truei. Although the latter two species exhibit similar ratios, examination of the first two columns in Table II reveals that in the absolute times for hemolysis in glycerol and in thiourea they are readily differentiated.

Figure 1 records graphically the results which have been summarized in Table II. Each mouse investigated in the present study has been plotted with regard to erythrocyte hemolysis in glycerol (along the abscissa) and with regard to the glycerol/thiourea ratio (along the ordinate). The hollow rectangles enclose all the individuals within a species. This figure shows in a striking way that it may be possible to determine the species to which an individual belongs by the appropriate hemolysis tests. For example, at one stage in the course of these

experiments a colleague kindly provided two blood samples without revealing the species from which they had been obtained. Hemolysis tests provided the following results:

	Time in seconds for 75 per cent hemolysis at 20° C.		
	0.3M glycerol	0.3M thiourea	G/T ratio
Mouse No. 1	12.1	15.0	0.80
Mouse No. 2	8.5	12.8	0.67

Both mice were correctly identified as leucopus.

Some evidence of zoological relationship is apparent in the glycerol/thiourea ratios obtained in this study. As can be noted in Table II, the ratios for leucopus, gossypinus and truei which are placed in the same taxonomic subgenus are all near one as a constant, while the ratio for eremicus which is placed in another subgenus is near two.

At the inception of this investigation some disconcerting variations in hemolysis times occurred within each species of Peromyscus. This led to an investigation of the effect of storage upon the rate of hemolysis of the red cells. In order to obtain a sufficient quantity for this purpose, it was necessary to use pooled blood of each Peromyscus species, whereas blood from individual guinea pigs was employed. Otherwise all blood samples were treated identically. Figure 2 shows the typical effect of storage upon the hemolysis rates of the Peromyscus and guinea pig red cells. Days in storage are plotted against the hemolysis time in glycerol. The red cells of each of the species within the genus Peromyscus show a marked and continued increase in hemolysis time upon storage while the red cells of the guinea pig show very little change during the same period of storage. The reason for this interesting storage effect has not yet been determined.

Discussion

Physiological and biochemical studies of blood have produced results both of broad evolutionary interest and also of value in the field of animal classification and identification. The evolutionary significance of results obtained from the studies of the osmotic pressures of blood (Scott, 1916) is well recognized. The extensive work of Reichert and Brown (1909) on the crystallography of hemoglobin among different species has provided convincing evidence of biochemical relationships among animals in general accord with the accepted taxonomic classification. The versatile and rapidly expanding field of systematic serology (see Boyden, 1942) has been employed on the one hand in the study of the possible origin of vertebrates (Wilhelmi, 1942), and on the other hand, in the investigation of the genetic basis for biochemical differences in the serum and blood cells of species and species-hybrids (Irwin and Cole, 1936; Irwin and Cumley, 1942).

The present investigation has revealed that consistent and measurable differences in the rates of hemolysis of the erythrocytes among very closely related species can be employed successfully to differentiate one species from another. Especially with regard to glycerol penetration, confirming observations by Jacobs,

and with regard to the glycerol/thiourea ratio the results indicate zoological relationship in general agreement with the existing system of classification. Whether such agreement between morphological classification and rate of osmotic hemolysis will always hold among closely related species can be determined only by further investigation.

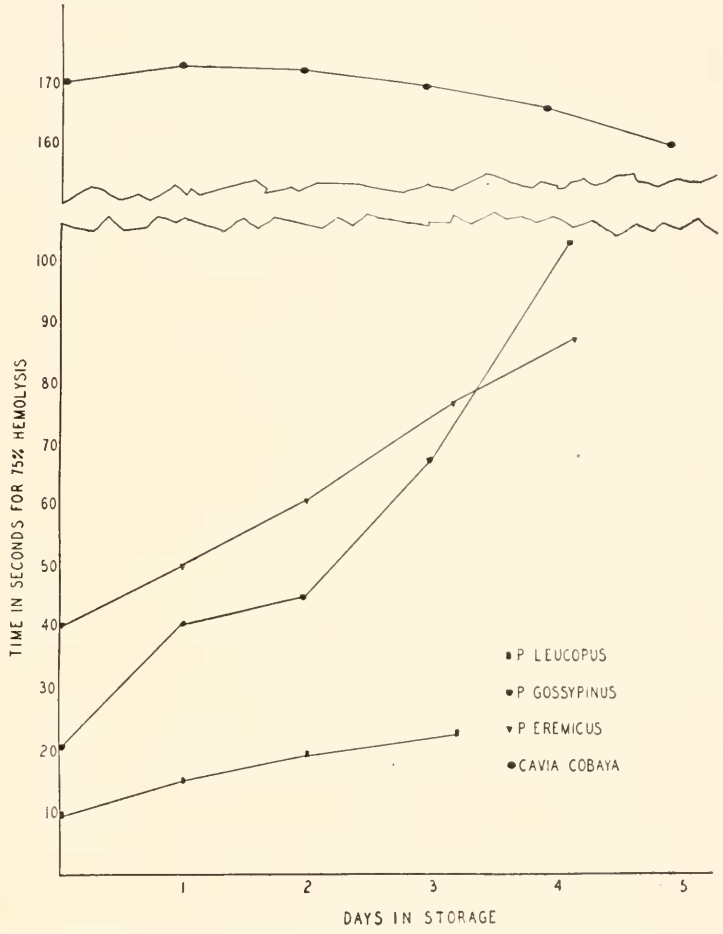

FIGURE 2. The effect of storage upon the rate of osmotic hemolysis (75 per cent) in 0.3 molar glycerol at 20° C. (Blood cells stored in 0.9 per cent NaCl at approximately 4° C.)

Preliminary studies on four offspring of a species-cross between leucopus and gossypinus indicate that these differences may be subject to genetic analysis although as yet the data are not sufficient for definite conclusions. Table III shows that in their hemolysis times in different substances the hybrid red cells are very similar to those of the leucopus parent stock while the values for the glycerol/thiourea ratio lie between those of the two parent stocks.

Specific differences in the properties of the cell membrane have introduced a

complicating feature to the problem of cell permeability, yet an understanding of the nature of such specific differences may go far towards a better understanding of the factors determining the permeability of the cell membrane in general. In the meantime collection of further data on species differences in erythrocyte permeability will serve the useful purpose of developing a physiological means of animal identification.

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TABLE III

Comparison of hemolysis times and G/T ratios of a species hybrid and its parent stocks

Species	Time in seconds for 75 per cent hemolysis at 20° C. in 0.3M				G/T ratio
	Ethylene glycol	Glycerol	Erythritol	Thiourea	
**P. leucopus noveboracensis*	5.6	9.5	31.8	13.3	0.71
**P. gossypinus palmarius*	7.1	22.1	110.0	19.4	1.14
leucopus-gossypinus hybrids	4.8	8.0	28.0	8.6	0.93
	5.1	10.4	39.0	10.1	0.97
	5.2	10.2	34.0	10.5	1.03
	4.9	7.8	20.0	8.7	1.12

* Average of the species.

SUMMARY

The erythrocytes of four species of mice within the genus Peromyscus were studied with regard to their rates of osmotic hemolysis in ethylene glycol, glycerol, erythritol and thiourea. Consistent species differences in hemolysis times were demonstrated by which it was possible in the case of the individuals studied to identify each species with certainty. Evidence of zoological relationship was apparent in the results.

Refrigeration storage of Peromyscus erythrocytes resulted in progressively decreased rates of hemolysis. Storage of Cavia (guinea pig) erythrocytes had very little effect upon their rates of hemolysis.

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